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RESEARCH ARTICLE

Enhanced antitumor activity of a combination of *MBD2*-antisense electrotransfer gene therapy and bleomycin electrochemotherapy

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Abstract

Background MBD2 is a methylated DNA-binding protein that has been previously suggested to have transcriptional silencing as well as DNA demethylase activities. We have previously shown that electrotransfer of an MBD2-antisense encoding plasmid inhibits tumor growth *in vivo*. In this study we tested whether a combination of *MBD2*-antisense gene therapy and bleomycin chemotherapy has an augmented antitumor effect in comparison with either monotherapy.

Methods Mice bearing human non-small-cell lung carcinoma line H1299 xenoplants were treated with electrotransfer of either bleomycin or MBD2-antisense expression plasmid or a combination of both therapies and tumor growth following treatment was monitored.

Results A combination of electrotransfer of *MBD2*-antisense and bleomycin electrochemotherapy has an additive inhibitory effect on the rate of tumor growth and a synergistic effect on the number of tumor-free animals when compared with either monotherapy.

Conclusions Our results suggest that a combination of *MBD2*-antisense electrotransfer gene therapy and chemotherapy with bleomycin is a candidate new approach to anticancer therapy. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords methylated DNA binding protein 2; demethylase; electrochemotherapy; bleomycin; tumorigenesis; antisense; electrotransfer gene therapy

Introduction

DNA methylation is an important component of the epigenome, which plays a critical role in programming gene expression [1]. A long list of data has implicated aberrations in the epigenome in cancer [2]. Tumor suppressor genes are methylated [2] while other regions of the genome are hypomethylated in cancerous tissue in comparison with their paired normal tissue [3]. While there is no simple correlation between the level of expression of the different proteins of the DNA methylation machinery and the changes in DNA methylation observed in cancer, some of these proteins are critical for tumorigenesis and their inhibition reverses tumor growth [4]. Antisense [5] or genetic [6] knockdown of DNA methyltransferase 1 (DNMT1), the enzyme that catalyzes the replication of the DNA methylation

pattern, reverses tumorigenesis. DNMT1 has been therefore proposed to be an anticancer target [4]. An antisense inhibitor of DNMT1, MG98, is now being tested in phase II clinical trials. Another member of the DNA methylation machinery that we have recently shown to be required for tumorigenesis is the methylated DNA binding protein MBD2 [7]. MBD2 is a methylated DNA-binding protein that has been proposed to act both as a suppressor of expression of methylated genes [8] as well as a demethylase, an enzyme that actively demethylates DNA [9]. Whereas earlier data failed to confirm the demethylase activity of MBD2, recent work from our laboratory shows that MBD2 demethylates ectopically methylated DNA in a promoter-specific manner [10]. Both putative actions of MBD2 are potentially important for maintaining the aberrant epigenome of transformed cells. Suppression of methylated genes might be required for silencing tumor suppressor genes whereas demethylation can activate tumor-promoting genes [11].

Antisense MBD2 gene therapy by either plasmid electrotransfer or adenoviral delivery inhibits anchorage independent growth of a number of human cancer lines *in vitro* as well as tumor growth *in vivo* [7]. One possible approach to take advantage of novel anticancer gene therapy targets is to combine them with classical chemotherapy agents. Combining gene therapy and traditional chemotherapy that act through different mechanisms of action could result in increased efficacy, resulting from either the additive or the synergistic effects of the two mechanisms of action, or from reduced toxicity and resistance. In this study, we tested this hypothesis by comparing the antitumor activity of a combination of electrotransfer of *MBD2*-antisense and the chemotherapeutic agent bleomycin with a single treatment with this agent. Bleomycin is an antineoplastic agent produced by fermentation from *Streptomyces verticillus* [12], which causes scission of both single- and double-stranded DNA breaks [13]. Human tumor xenografts resistance to bleomycin has been shown to be associated with rapid drug metabolism and decreased accumulation [14]. Administration of bleomycin by cell electropermeabilization significantly increases its accumulation in tumors and its antitumor activity [15,16]. This therapeutic technology has been introduced as electrochemotherapy (for reviews, see [17,18]). Since bleomycin damages the genome whereas *MBD2*-antisense targets the epigenome, we reasoned that the combination of these drugs acting by a different mechanism might result in an augmented antitumor activity. The results presented here support the hypothesis that a combination of *MBD2*-antisense electrotransfer gene therapy and bleomycin electrochemotherapy, using a single treatment with bleomycin and five treatments with *MBD2*-antisense plasmid, results in a considerable increase in antitumor activity as compared with treatment with a single agent. This combination is proposed as a potential new modality for anticancer therapy.

Materials and methods

H1299 human tumor xenograft model

H1299, a human non-small-cell lung carcinoma cell line, was maintained as a monolayer in DMEM medium containing 10% fetal calf serum. The tumors were injected into the flanks of 6- to 8-week-old female Swiss nude mice and explants were passaged at least twice before each experiment. Explants of H1299 tumors (20 mm^3) were subcutaneously inoculated into the flank. The tumors were treated once they had reached a volume of 30–100 mm^3.

Sense and antisense MBD2b/demethylase plasmids

His-MBD2/demethylase sense and antisense expression plasmids have been described previously. Briefly, the cDNA encoding MBD2b (Genbank AF072242) was introduced at the Not I site of the multiple cloning site of the pCDNA3 vector (Invitrogen, Nederland) in either the sense or antisense orientation under the control of the CMV promoter and upstream to the late polyadenylation signal of SV40 simian virus (Genbank SV4CG). The expression of the cDNAs in tumors *in vivo* was previously validated.

Bleomycin A2 sulfate

Bleomycin A2 sulfate (RP23202 A, N′-bleomycinamide sulfate) was provided by Rhone-Poulenc Rorer (batch number 0428C9/291 020). Bleomycin was dissolved at a concentration of 0.5 mg/ml in a 150 mM NaCl solution.

In vivo electrotransfer of plasmids and bleomycin electrochemotherapy

For plasmid electrotransfer alone, the mice were injected intramuscularly (tibialis cranialis) with 50 µl of 150 mM NaCl 30 min prior to DNA injection. DNA plasmids (either MBD2/demethylase sense or antisense, 50 µg in 80 µl 150 mM NaCl) were injected five times in a 2–3-day interval between injections into the H1299 tumor implants using a Hamilton syringe and a 26 G needle. Both sides of the tumors were then covered with conductive gel and placed between two flat parallel stainless steel electrodes 0.45 cm apart. Then, 20–30 s after DNA injection, each tumor was submitted to eight pulses of 20-ms duration at a voltage/distance ratio of 500 V/cm, delivered at the frequency of 1 Hz, using an electropulsator PS 15 (Jouan, St Herblain, France). As a control, H1299 tumors were submitted to similar electric pulses after 150 mM NaCl injection.

Between 10–13 tumors were injected for each treatment (see Table 2 for details of the different experiments).

In these described experiments, group 2 (bleomycin 25 μg) and groups 3 and 5 (MBD2 antisense gene therapy without or with 25 μg bleomycin, respectively) were performed twice. Group 1 (NaCl + electrotransfer) was used as a control since we have already shown that there was no difference between untreated mice and NaCl + electrotransfer treated mice [7]. Group 4 (*MBD2*-sense) has also been repeated in another set of experiments (data not shown). Group 6 (*MBD2*-sense + 25 μg bleomycin) has been performed only once. The tumor growth was evaluated with time by measuring the volume (in mm^3) of each tumor following the formula: (length × width × height)/2. The tumor growth curve was expressed as either mean volume ± SEM or as median volume as a function of time.

To evaluate the benefit of the combination of gene therapy and chemotherapy, the first injection and electrotransfer of MBD2-antisense plasmid (50 μg DNA/80 μl 150 mM NaCl) was preceded 30 min earlier by bilateral intramuscular (tibialis cranialis) injection of 50 μl of 150 mM NaCl containing 25 μg bleomycin (i.e. injection of 25 μl in each tibialis cranialis muscle). To determine the effect of bleomycin monotherapy, 25 μg of bleomycin in 50 μl 150 mM NaCl were injected into the tibialis cranialis muscles and, 30 min later, each tumor was submitted to eight pulses of 20-ms duration at a voltage/distance ratio of 500 V/cm, delivered at the frequency of 1 Hz as already described.

Results

Comparison of the therapeutic effects of a combination of *MBD2*-antisense and bleomycin electrotransfer on H1299 tumor growth *in vivo*

We have previously shown that *MBD2*-antisense electrotransfer inhibits the growth of human tumor xenografts in mice [7]. Delivery of electric pulses has been previously shown to enhance bleomycin intratumoral cell entry and antitumor effect [15,16]. A plasmid bearing the *MBD2* cDNA in the sense orientation was used as a control for the *MBD2*-antisense plasmid. The scheme in Figure 1 depicts the schedule of injections for the different treatment groups. The average tumor volumes were determined for each treatment group at the time points indicated in Figure 2. The growth curves of the tumors presented in Figure 2 show that either *MBD2*-antisense or bleomycin treatment alone inhibits tumor growth relative to their control. However, a combination of bleomycin and *MBD2*-antisense treatment inhibits tumor growth to a larger extent than either single treatment.

In Table 1, we compare the number of days that it takes the tumors to reach a volume of 1000 mm^3 under different treatment conditions. NaCl-treated tumors reach the average volume of 1000 m^3 21 days post-initiation of treatment and *MBD2*-sense-treated tumors reach the same volume by 24 days. Single therapy with *MBD2*-antisense delays tumor growth by 18 days

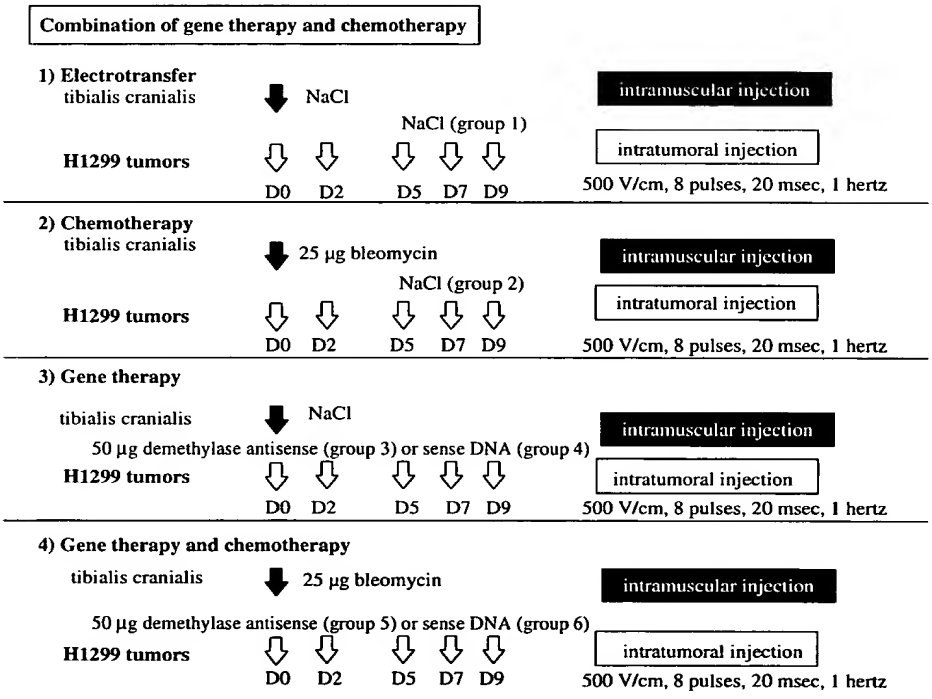

Figure 1. Combination and monotherapy treatment protocols. The schedules of electrotransfer of NaCl solution, plasmids and bleomycin for the different treatment groups are indicated

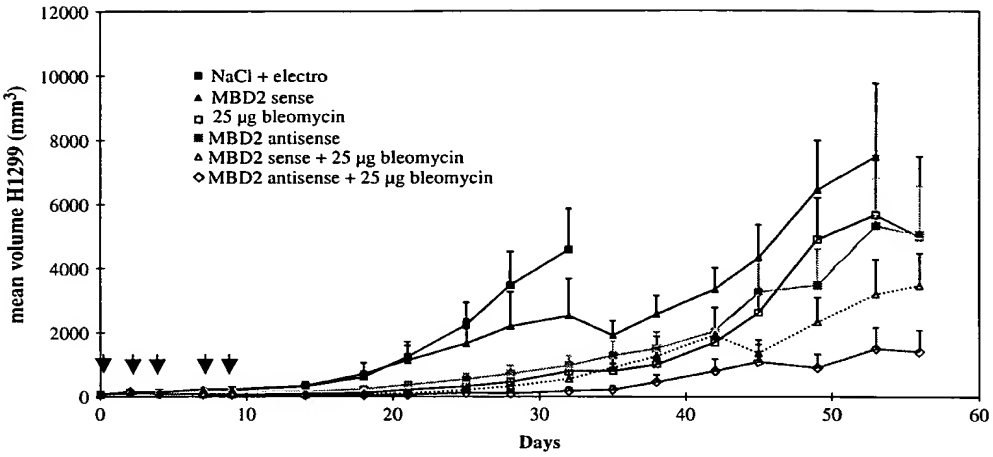

Figure 2. Inhibition of tumor growth by a combination therapy of bleomycin and *MBD2*-antisense. Electrotransfers of either a NaCl solution alone, or with 50 µg of either, His-Mbd2-sense or His-MBD2-antisense, 25 µg bleomycin alone or with MBD2 expression plasmids in either orientations (50 µg) were performed on xenograft H1299 tumors in nude mice as described in Materials and methods. Arrows indicate time points at which electrotransfer was performed. Volumes (mm^3) are plotted against time up to 56 days post-implantation. 10–13 animals per condition were used and results are plotted as means ± SEM

Table 1. Latency to reach a tumor volume of 1000 mm^3 in the different treatment groups and statistical comparison of the risk to reach 1000 mm^3 of the different treatment groups using either Student's t-test or the log-rank Kaplan-Meier test

	Day 1000 mm^3 (median)
Group 1: NaCl	20.9
Group 2: 25 µg bleomycin	38.0
Group 3: DNA demethylase antisense	38.6
Group 4: DNA demethylase sense	24.3
Group 5: DNA demethylase antisense + 25 µg bleomycin	52.0
Group 6: DNA demethylase sense + 25 µg bleomycin	40.3

Statistical comparison	Student t-test mean comparison		Log-rank Kaplan-Meier risk to reach 1000 mm^3 tumor volume	
DNA demethylase antisense versus NaCl electrotransfer	$p = 0.0201$	*	$p = 0.0029$	**
25 µg bleomycin electrotransfer versus NaCl electrotransfer	$p = 0.0008$	***	$p = 0.0001$	***
DNA demethylase sense versus NaCl electrotransfer	$p = 0.2667$	NS	$p = 0.2803$	NS
DNA demethylase antisense + 25 µg bleomycin versus 25 µg bleomycin electrotransfer	$p = 0.0088$	**	$p = 0.0056$	**
DNA demethylase sense + 25 µg bleomycin versus 25 µg bleomycin electrotransfer	$p = 0.2842$	NS	$p = 0.1750$	NS
DNA demethylase antisense + 25 µg bleomycin versus NaCl electrotransfer	$p = 0.0001$	***	$p < 0.0001$	***
DNA demethylase sense + 25 µg bleomycin versus NaCl electrotransfer	$p = 0.0002$	***	$p < 0.0001$	***

whereas single therapy with bleomycin delays it by 17 days. A combination therapy of bleomycin and *MBD2*-antisense delays tumor growth by 31 days relative to control animals. A combination of bleomycin and the *MBD2*-sense plasmid had some effect as well in combination with bleomycin, but this effect was less marked than the one observed with *MBD2*-antisense: 19 days delay vs. 31 days with the antisense plasmid. A statistical analysis of this experiment shown in Table 1 reveals that there is no statistically significant difference between *MBD2*-sense therapy and the control, while there is a statistically significant difference between *MBD2*-antisense and the control. There is a significant difference between the combination treatment with bleomycin vs. *MBD2*-antisense and bleomycin monotherapy whereas the difference between a combination of *MBD2*-sense and bleomycin vs. bleomycin monotherapy is not significant. These data support the conclusion that there is an additive effect of combining bleomycin and *MBD2*-antisense therapy.

MBD2-antisense/bleomycin combination therapy reduces tumor incidence

While an analysis of average tumor volumes shows an additive effect of *MBD2*-antisense and bleomycin therapy, an analysis of tumor-free animals in each group presented in Table 2 indicates a synergistic effect of the two therapies. As shown in Table 2, either *MBD2*-antisense or bleomycin (25 μg/ml) monotherapy results in a slight increase in incidence of tumor-free animals (10%) compared with the other control groups. The combination therapy reduced incidence of tumors by up to 40%. In summary, our results support some synergism between *MBD2*-antisense and bleomycin in reducing tumor incidence.

Discussion

Combination therapy of genetic medicine and traditional chemotherapy is emerging as an important modality in anticancer therapy. The advantages of this form of therapy are obvious since they can help reduce the toxicity of chemotherapeutic agents, as well as mitigate the risk of treatment with novel and relatively uncharted genetic therapies by supplementing them with known chemotherapeutic agents. This approach is especially important for clinical trials assessing new genetic therapies.

In this study, we compared the antitumor activity of a combination of electrotransfer of *MBD2*-antisense and the chemotherapeutic agent bleomycin with a single treatment with this agent. In the experiment presented here, bleomycin was injected into a skeletal muscle and electrotransfer was applied 30 min later, following a previously reported protocol by Mir *et al.* [16]. The chosen drug administration route is not unusual, although clinical trials currently use intratumoral bleomycin injections for curative treatments. Both systemic bleomycin delivery (intravenous, intraperitoneal or intramuscular) and intratumoral delivery have been largely used in mouse experiments as well as in early clinical studies (see [17,18]; for reviews, see [19]), giving excellent results. The advantages of both administration routes are discussed in [17]. For example, systemic administration of bleomycin might be suitable if a large number of nodules have to be treated, or when injection in the tumor mass is almost impossible (like in pancreatic carcinomas). The time necessary for the bleomycin to reach the tumor is 30 min [16], and allows a homogenous distribution of the drug in the tumor vasculature. Furthermore, injecting bleomycin intratumorally might have led to a complete response, masking the potential effects of gene therapy. As a partial response of bleomycin was required to determine the additional effects of gene therapy, we chose an intermediate dose of 25 μg of bleomycin per mouse (corresponding to 1.25 mg/kg). Preliminary experiments have shown that a 5 μg dose had a small effect while 25 μg led to a better partial response (data not shown).

Based on the experiments with *MBD2*-antisense discussed previously [7], we performed several preliminary experiments to determine the effective dose response with the *MBD2*-antisense plasmid. Our preliminary results indicated that five intratumoral injections of 50 μg of plasmid followed by electrotransfer induced an antitumorigenic effect, whereas three or five injections of 20 μg of plasmid did not show any activity (data not shown). Therefore, we decided to study the combination of MBD2 gene therapy (five injections of 50 μg of plasmid) with electrochemotherapy (25 μg per mouse). We also noticed that the transfection efficiency following a single *in vivo* electrotransfer was low (data not shown). This is consistent with other reports in the literature, which range from about 4% with B16 Bl6 tumors [20], to 8% with B16 F10 tumors [21]. As electrotransfer is simple to perform and does not induce immune response, it was repeated, resulting in increased accumulation of transfected cells. Expression of transgenes in tumors is relatively short term: high expression of β-galactosidase was observed at day 3 [22]; Il-12 and IL-18 expression after plasmid electrotransfer peaked around day 4 [21,23]. Therefore, to obtain good antitumor effects after electrotransfer gene therapy, several repetitions of electrotransfer twice a week [22] or at 2-day intervals were previously reported [24]. Based on this reasoning we chose to inject 50 μg of *MBD2*-antisense plasmid five times at intervals of 2–3 days (see Figure 1).

We show here that a combination of bleomycin and *MBD2*-antisense gene therapy augments the antitumor activities of either agent. Combination therapy might result in either an additive effect when the two agents act on separate pathways or a synergistic effect when they act on the same or interacting pathways. It is expected that bleomycin and MBD2 inhibition act on separate pathways since bleomycin induces DNA damage and is cell cycle specific and appears to inhibit cell progression out of G2 phase [25], whereas MBD2 is involved in regulation of the epigenome either by suppressing methylated genes [8] or by inducing demethylation and activation of methylated genes [10]. Inhibition of MBD2 does not inhibit cell cycle progression [7]. The additive effect of the combination

Table 2. Combination of bleomycin and *MBD2*-antisense therapy reduces tumor incidence. The number of tumor-free animals after 56 days post-implantation is indicated (tumor-free/total number of tumor-bearing animals). Experiments 1 and 2 refer to two independent experiments

	Experiment 1	Experiment 2
NaCl/electrotransfer (group 1)		0/11
Bleomycin 25 μg (group 2)	1/13	1/11
Demethylase antisense (group 3)	0/13	1/10
Demethylase sense (group 4)		0/11
Bleomycin 25 μg/demethylase antisense (group 5)	3/11	4/10
Bleomycin 25 μg/demethylase sense (group 6)		0/10

therapy on retarding tumor growth is consistent with the different mechanism of action of these two therapies. The synergistic effect that the combination has on reduction in tumor incidence suggests, however, that there is some positive interaction between the antitumor mechanisms of action of the two therapies. Further experiments and understanding of the mechanism of action of MBD2 are required to understand how bleomycin and inhibitors of MBD2 synergistically interact in reducing tumor incidence.

We also noticed that the *MBD2*-sense plasmid also had some antitumoral effect, which is augmented in combination with bleomycin. We do not know as yet what are the mechanisms stimulated by overexpression of MBD2 in tumor cells. It is therefore difficult to compare the effects of *MBD2*-antisense with the effect of MBD2 overexpression. We do not know if the difference in effects between these two treatments should be of significance. We believe that the effect of the *MBD2*-sense plasmid is not unexpected. Nevertheless, we believe that the fact that 7 mice out of 21 in the treated group (33%) showed complete long-term regression of tumors in the MBD2 antisense + bleomycin group, and no mice at all showed complete regression in the sense + bleomycin treated group, is of significance.

Nevertheless, the experiments reported in this paper suggest that a combination of bleomycin electrochemotherapy and electrotransfer of *MBD2*-antisense gene therapy is a promising candidate anticancer therapy and that other combinations of chemotherapy and MBD2 inhibition should be tested in the future as potential anticancer therapies. More generally, the combination of electrochemotherapy and gene therapy seems of interest in cancer treatment. This has already been suggested in the case of GM-CSF or Il-2 expression plasmids combined with bleomycin [26] or with p53 expression plasmid combined with an anticancer agent [27]. Since electrochemotherapy is very effective on primary tumors, and there is currently no efficient method of preventing metastases, one might think that the combination with gene therapy should focus on preventing metastases. Therefore, further studies should aim at combining electrochemotherapy and antimetastatic gene therapy. Cytokines such as Il-12 play a role in inducing an immune response against cancer cells, and have proven efficient in antitumor gene therapy [21,23]. Several gene therapy experiments have already shown some antimetastatic effects: for example, suicide gene therapy using *HSVtk*/ganciclovir technology suppressed the growth and metastasis of subcutaneously grafted mammary tumors in mice, although no complete regression was noted [22]. Antiangiogenic gene therapies have also proven useful in preventing metastasis, and offer great hopes, as, for example, a gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2 [28] or electrotransfer of a endostatin-encoding plasmid [29]. In the latter case, the plasmid can be transfected into muscle cells since endostatin is a circulating protein. This would allow an efficient systemic gene therapy since the skeletal muscle is more easily transfected than tumors combined with a classical electrochemotherapy. Intratumoral electrotransfer of *TRAIL/Apo2* ligand, an apoptosis inducer, also showed inhibition of spontaneous lung metastasis in a hepatocellular carcinoma model, in addition to inhibition of tumor growth [30]. Further studies should determine whether the combination of electrochemotherapy and gene therapy is potentially promising for treatment of tumors as well as metastases.

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